Betaine as a Determinant of Postmethionine Load Total Plasma Homocysteine Before and After B-Vitamin Supplementation

Pål I. Holm, Øyvind Bleie, Per M. Ueland, Ernst A. Lien, Helga Refsum, Jan E. Nordrehaug, Ottar Nygård

Objective—Betaine is a substrate in the betaine–homocysteine methyltransferase reaction, converting homocysteine to methionine. There are only sparse data on plasma betaine as a determinant of the plasma total homocysteine (tHcy) concentration.

Methods and Results—Ninety patients undergoing coronary angiography were randomized into 4 groups administered oral: (1) folic acid (0.8 mg), vitamin B12 (0.4 mg), and vitamin B6 (40 mg); (2) folic acid and vitamin B12; (3) vitamin B6 alone; or (4) placebo. Nonfasting blood samples were collected at baseline and 3, 14, and 28 days and 3, 6, and 12 months after treatment start. A 4-hour methionine-loading test (0.1 g/kg) was performed at baseline and after 3 months. At baseline, median (interquartile range) plasma betaine was 36.9 μmol/L (range: 30.3 to 46.8) and was increased by 15% after methionine loading. The postmethionine load (PML) increase in tHcy was inversely related to plasma betaine ($\beta = -0.29, P = 0.02$) and even more strongly to PML betaine ($\beta = -0.47, P < 0.001$). After 3 months of intervention, the relation between the PML increase in tHcy and PML betaine was weakened ($\beta = -0.33, P = 0.007$).

Conclusions—Plasma betaine is a strong determinant of the PML increase in tHcy in subjects not supplemented with B-vitamins. (Arterioscler Thromb Vasc Biol. 2004;24:301-307.)

Key Words: betaine ■ homocysteine ■ folate ■ vitamin B12 ■ vitamin B6

Homocysteine is an established risk factor for occlusive vascular disease. High plasma concentration is also associated with impaired cognitive function, Alzheimer disease, adverse pregnancy outcomes, and congenital malformations, particularly neural tube defects.

The plasma concentration of total homocysteine (tHcy) is determined by a variety of physiological, lifestyle, and genetic factors and disease states. Among these, renal function, folate, and cobalamin status are particularly influential. The vitamin effects are explained by the functions of 5-methyltetrahydrofolate as substrate and cobalamin as cofactor in the ubiquitous methionine synthase reaction, which catalyzes the remethylation of homocysteine to methionine. Vitamin B6, however, has no or a moderate effect on plasma tHcy.

Methionine loading, which involves measurement of tHcy after a standard oral methionine dose, was originally designed to reveal defects in the vitamin B6-dependent transsulfuration pathway. Plasma postmethionine load (PML) tHcy has since been associated with cardiovascular risk independent of basal tHcy. Folate, as well as vitamin B6 status, are significant predictors of PML tHcy. These vitamin effects in humans are at variance with the idea, based on enzymological data, that PML tHcy is determined by the activity of vitamin B6-dependent enzyme, cystathionine β-synthase, whereas the folate-dependent homocysteine remethylation regulates basal tHcy.

Betaine is (trimethylglycine) is obtained in small amounts through the diet or is generated in liver and kidney from choline through the sequential action of choline oxidase (EC 1.1.3.17) and betaine-aldehyde dehydrogenase (EC 1.2.1.8). Betaine serves as methyl donor of the zinc metalloenzyme, betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5). This enzyme is mainly confined to the liver and kidney and catalyzes an alternative route of homocysteine remethylation to methionine (Figure 1). The role of betaine as a methyl donor explains the observations that betaine supplementation reduces tHcy in homocystinurics and in healthy individuals. Betaine also attenuates the tHcy increase after methionine loading in healthy subjects and in cardiovascular and renal patients. However, except for a recent Canadian study reporting on an inverse relation between fasting tHcy and betaine in cardiovascular patients, no study has addressed the question of the role of endogenous betaine as a determinant of basal and PML tHcy.
Recruited patients were randomized into 4 groups in a 2 × 2 factorial block design. Group FB (n=22) was administered daily folic acid (0.8 mg), vitamin B12 (cyanocobalamin, 0.4 mg), and vitamin B6 (pyridoxine, 40 mg). Group F (n=23) was administered folic acid and vitamin B12. Group B (n=21) was administered vitamin B6. Group P (n=24) was administered placebo. For the first 2 weeks, the groups (FB and F) receiving folic acid were administered an additional loading dose of folic acid (5 mg/d). Packages of trial tablets were prepared and given serial number in random order in blocks of 20 by Alpharma A/S (Copenhagen, Denmark). Compliance monitoring was accomplished by tablet counting and by determination of vitamin B concentrations in plasma.

The subjects underwent a full routine medical examination at baseline before coronary angiography. Coronary angiography was performed 3 days after starting vitamin therapy.

Blood Collection and Biochemical Analyses
Nonfasting (basal) blood samples were collected at baseline, after 3 days, at 2 and 4 weeks, and at 3, 6, and 12 months of B-vitamin intervention. A methionine loading test (0.1 g/kg body weight) was performed at baseline and after 3 months. PML blood samples were drawn at 4 hours after loading. EDTA blood samples were immediately placed on ice, centrifuged within 30 minutes, and EDTA plasma stored at −80°C until analysis.

Routine blood analyses, including serum creatinine, were analyzed at the Central Laboratory of Haukeland University Hospital on Technicon Chem 1 (Bayer, Leverkusen, Germany). The tHcy,23 betaine, choline, di-MethylGlyoxime (DMG),22 cobalamin24 plasma folate,25 and pyridoxal phosphate26 were analyzed by published methods.

Statistics
Because of skewed distribution of values for tHcy and some metabolites, data are presented as median values with interquartile ranges. Associations between variables were evaluated by Spearman rank correlation and multiple linear regression analyses. Variables considered likely to influence the outcome parameters were included in the multivariate models. Mann-Whitney U test or Kruskal-Wallis test was used for between-group comparisons of continuous variables, and Wilcoxon signed rank test was performed for comparison within groups at baseline and after 3 months. Data were analyzed using SPSS 11.0 (SPSS Inc.).

Results
Subject Characteristics and Blood Indices at Baseline
A total of 90 patients were included. Their median age was 62 years (range 38 to 80); 77% were males. Blood indices at baseline for the whole study group are given in Table 1. Median betaine was 36.9 μmol/L and median PML betaine was 44.5 μmol/L. One patient (in group P) had plasma cobalamin (117 pmol/L) below the lower reference limit (200 pmol/L), along with elevated MMA (0.77 μmol/L), and was considered cobalamin-deficient. Plasma folate was below the lower reference limit (4.5 nmol/L) in 10 patients.

Table 1 also shows the expected marked increase in tHcy and also a moderate increase in median betaine (15%; P<0.001), choline (7%; P=0.001), and DMG (18%; P<0.001) after methionine loading.

The concentrations of basal and PLM betaine in particular, but also of choline and creatinine, were markedly higher in males than in females (Table 1). No parameters differed significantly according to treatment group, except basal tHcy (18% difference; P=0.04) and basal choline (15%; P=0.01) and PML choline (21%; P=0.04) (data not shown). Details on plasma pyridoxal phosphate26 and cobalamin27 in this population have been given in separate publications.
Betaine, Choline, and DMG According to Duration and Type of Intervention

Figure 3 depicts plasma concentrations for choline, betaine, and DMG measured at intervals from baseline to 12 months. PML concentrations at baseline and after 3 months are included. The levels for betaine in particular, but also for choline and DMG, were higher in men than in women assured after methionine loading showed similar relations to gender, age, creatinine, and the B-vitamins as observed for the basal concentrations (data not shown).

Betaine as a Determinant of PML tHcy

The ΔPML tHcy was inversely associated with basal betaine (model I), PML betaine (model II) (Table 3), and the PML increase in betaine (β = −0.27, P = 0.02) by multiple linear regression. PML betaine was a stronger predictor than basal betaine of ΔPML tHcy. The regression models also included age, sex, creatinine, and the B-vitamins, and these factors were not significantly related to ΔPML tHcy (Table 3). Additional adjustment for basal or PML choline and basal or PML DMG in the respective models had no effect (data not shown).

Determinants of Betaine, Choline, and Dimethylglycine

We assessed age, gender, creatinine, and the B-vitamins as predictors of basal betaine, choline, and DMG at baseline by multiple linear regression (Table 3). Betaine showed a strong relation to sex, whereas choline in particular, but also DMG, was positively related to creatinine. The metabolites were not significantly related to any B vitamin, ie, folate, cobalamin, or vitamin B6 (Table 3). Betaine, choline, and DMG measured before and after loading (PML), were significantly and positively correlated (Table 2; data for choline and DMG not shown).

Simple Correlations at Baseline

Simple correlations at baseline are shown in Table 2. Baseline tHcy was positively related to age, creatinine, and basal DMG, and were inversely related to plasma folate and cobalamin.

The increase in tHcy after methionine loading (ΔPML tHcy) was not related to the levels of B-vitamins. The ΔPML tHcy showed a strong inverse association with PML betaine (r = −0.37, P < 0.001) and a moderate association with basal betaine (r = −0.23, P = 0.03), as also depicted in Figure 2.

The concentrations of betaine, choline, and DMG, measured after methionine loading showed similar relations to gender, age, creatinine, and the B-vitamins as observed for the basal concentrations (data not shown).

**TABLE 2. Spearman Correlations at Baseline**

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Creatinine</th>
<th>ΔPML tHcy</th>
<th>Betaine</th>
<th>PML betaine</th>
<th>Choline</th>
<th>DMG</th>
<th>Folate</th>
<th>Cobalamin</th>
<th>Vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>tHcy</td>
<td>0.50†</td>
<td>0.44†</td>
<td>0.40†</td>
<td>0.07</td>
<td>0.07</td>
<td>0.18</td>
<td>0.32*</td>
<td>−0.21*</td>
<td>−0.21*</td>
<td>−0.08</td>
</tr>
<tr>
<td>ΔPML tHcy</td>
<td>0.14</td>
<td>0.08</td>
<td>−0.23*</td>
<td>−0.37†</td>
<td>0.01</td>
<td>0.03</td>
<td>0.06</td>
<td>−0.13</td>
<td>0.02</td>
<td>0.00</td>
</tr>
<tr>
<td>Betaine</td>
<td>0.13</td>
<td>0.07</td>
<td>0.79†</td>
<td>0.49†</td>
<td>0.42†</td>
<td>0.06</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PML betaine</td>
<td>0.15</td>
<td>0.18</td>
<td>0.34*</td>
<td>0.33*</td>
<td>0.50†</td>
<td>0.05</td>
<td>−0.12</td>
<td>−0.19</td>
<td>−0.09</td>
<td></td>
</tr>
<tr>
<td>Choline</td>
<td>0.17</td>
<td>0.28*</td>
<td></td>
<td></td>
<td></td>
<td>−0.14</td>
<td>−0.1</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>DMG</td>
<td>0.28*</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*P < 0.05.
†P < 0.001.

ΔHcy indicates total homocysteine; ΔPML, postmethionine load increase; DMG, dimethylglycine.
throughout the intervention period and showed a transient increase after methionine loading (Figure 3).

We compared the basal and PML concentrations of betaine, choline, and DMG at 3 months according to the treatment groups FB, F, B, and P. The metabolite concentrations, except PML choline \( (P=0.03) \), were not significantly different between the groups \( (P=0.4) \) (data not shown).

**B-Vitamin Supplementation and the Betaine–PML tHcy Relationship**

After 3 months of intervention, plasma cobalamin and plasma folate were higher than 277 pmol/L and 25 nmol/L, respectively, in all patients in groups FB and F, and pyridoxal phosphate was higher than 142 nmol/L in groups FB and B. No significant changes in median levels of these vitamins occurred in the nonsupplemented groups.

\( \Delta \text{PML tHcy} \) (\( \mu \text{mol/L} \); median [interquartile range]) decreased in the groups FB (from 19.4 [15.5 to 22.7] to 14.7 [11.6 to 17.2], \( P=0.004 \)), F (from 19.7 [16.6 to 24.6] to 15.8 [12.9 to 18.9], \( P=0.002 \)), and B (from 19.3 [15.3 to 23.6] to 16.3 [13.5 to 17.9], \( P=0.005 \)) given B-vitamins. \( \Delta \text{PML tHcy} \) did not change in the placebo group P (from 20.9 [17.0 to 30.0] to 21.8 [16.3 to 30.2], \( P=0.5 \)).

After 3 months of intervention, the inverse association between \( \Delta \text{PML tHcy} \) and PML betaine in the subjects receiving B-vitamins was essentially abolished but was still present in the subgroup (P) receiving placebo, as depicted in Figure 2. Multiple linear regression analyses of the association between \( \Delta \text{PML tHcy} \) and PML betaine in the separate intervention groups demonstrated that the association was absent in the groups FB and F administered folic acid \( (P>0.6) \), showed a trend in the group B administered vitamin B6 \( (P=0.09) \), and was still strong and significant in the group P administered no B-vitamins \( (\beta=-0.66, P=0.02) \). All these models were adjusted for age, sex, creatinine, cobalamin, folate, and vitamin B6.

Predictors of \( \Delta \text{PML tHcy} \) at 3 months in the whole study group were assessed by multiple regression (Table I, available online at http://atvb.ahajournals.org). The model contained the B-vitamins, in addition to creatinine, age, and sex.
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Study Design
The strength of the study is its longitudinal, prospective design, which allows comparison of betaine effects before and after B-vitamin intervention in the same individuals. It was clearly shown that in the combined group treated with B-vitamins, the betaine-PML-tHcy association was essentially abolished, but the small sample size provided limited statistical power to delineate the effects of individual B-vitamins.

We measured PML tHcy 4 hours after methionine ingestion. This short interval was chosen for logistic reasons. A recent study demonstrated that at this time point, PML tHcy has higher within-subject and between-subject coefficients of variation (CV) than the PML tHcy measured at 6 or 8 hours after methionine intake, possibly because of variable methionine absorption a short time after loading. Furthermore, we observed that betaine showed a stronger association with ΔPML tHcy than with PML tHcy. However, analytical CV increases when PML tHcy is adjusted for basal tHcy to obtain ΔPML tHcy, because the variance has the property of being additive. Thus, increased biological and analytical variations may actually lead to underestimation of the “true” association between betaine status and PML tHcy.

Concentrations of Betaine, Choline, and Dimethylglycine
The median plasma concentration of betaine reported here (∼37 μmol/L, Table 1) is similar to the concentration previously reported by us in healthy blood donors and by others. We observed a strong relation between betaine and gender, with higher levels in males than in females (Table 1, Figure 3), which has also been reported by others. The presence of consensus sites for steroid hormones, including estrogen and androgen binding sites, in the human betaine homocysteine methyltransferase gene may represent a molecular basis of sex steroid effects on plasma betaine.

We observed a median concentration of plasma choline (9 μmol/L) that is equal to the concentration (∼10 μmol/L) previously measured in healthy subjects. The median plasma DMG (3.6 μmol/L) is somewhat higher than the DMG levels (∼2 to 3 μmol/L) previously reported by us and others. Slightly elevated plasma DMG may be related to the possibility that the cardiovascular patients have moderately impaired renal function, which is known to affect the DMG concentration. Conceivably, methodological difference may also account for different metabolite (DMG) levels between various study populations.

Human Studies on Betaine and tHcy
The strong inverse association between ΔPML tHcy and plasma betaine, particularly PML betaine, in subjects not receiving B-vitamin supplementation is the most important result of the present study. The data are in agreement with consistent observations that betaine supplementation reduces PML tHcy and also fasting tHcy in healthy subjects and tHcy in renal patients and in homocystinurics. However, our data (Table 2) do not confirm a recent observation of a significant inverse relation between (basal) plasma betaine and tHcy in 122 Canadian cardiovascular patients.

Discussion
We present data on the relation between betaine and plasma tHcy in 90 patients with cardiovascular disease before and during long-term intervention with folate, cobalamin, and vitamin B6, as implemented in the WENBIT. We demonstrate, for the first time to our knowledge, that the increase in tHcy after methionine loading (∆PML tHcy) was determined by PML betaine. Basal betaine was a weaker predictor. In 68 subjects supplemented with B-vitamins for 3 months, ∆PML tHcy was reduced, and its inverse relation to betaine was weakened, particularly in the subgroups administered folic acid.
Mechanisms and Role of Betaine in Homocysteine Remethylation

Methionine loading caused a 3-fold increase in tHcy, a 15% increase in plasma betaine and DMG, and also a significant increase in choline (Table 1, Figure 3). The choline response may reflect enhanced supply of choline for the synthesis of betaine or a choline-sparing effect from superfluous methionine. The elevation of plasma betaine and DMG levels, however, indicates an increased flux through the BHMT pathway, which is in accordance with the observation that ∆PML tHcy is strongly and inversely related to PML betaine and also to PML increase in betaine. Thus, increased metabolic flux through the choline-betaine-DMG pathway may occur in response to high levels of homocysteine and/or methionine.

After treatment with combinations of folic acid, vitamin B12, and vitamin B6 for 3 months, the relation between PML betaine and ∆PML tHcy was weakened in the whole study group and was abolished in the subgroups administered folic acid. This indicates that enhancement of folate-dependent remethylation catalyzed by methionine synthase downregulates the BHMT reactions and suggests a cross-talk between these two pathways.

The significance of betaine in homocysteine homeostasis is not clear, but the view prevails that the BHMT mainly functions to conserve homocysteine under conditions of methionine deficiency. This assumption is based on animal experiments demonstrating a dramatic induction of BHMT during methionine deficiency in combination with excess dietary choline. Our observation of an inverse relation between betaine and ∆PML tHcy indicates an additional function of the BHMT pathway, i.e., reduction of the homocysteine increase after methionine intake. This idea is difficult to reconcile with the fact that BHMT is inhibited by S-adenosylmethionine but gains some support from experiments in rats, demonstrating a moderate net increase in BHMT activity during methionine deficiency in combination with excess dietary choline. 

Our observation of an inverse relation between betaine and ∆PML tHcy indicates an additional function of the BHMT pathway, i.e., reduction of the homocysteine increase after methionine intake. This idea is difficult to reconcile with the fact that BHMT is inhibited by S-adenosylmethionine but gains some support from experiments in rats, demonstrating a moderate net increase in BHMT activity during methionine deficiency in combination with excess dietary choline. This metabolic effect contrasts to the result of the vitamin B6-dependent cystathionine-β-synthase reaction, which irreversibly directs superfluous homocysteine into the transsulfuration pathway.

Implications and Conclusion

The data presented here indicate that betaine through the BHMT reaction buffers and tends to reduce homocysteine during methionine excess. This reaction, which conserves the homocysteine carbon backbone, may be beneficial under conditions of limiting supply of folate required for enhancement of the methionine synthase reaction. These findings emphasize the complementary relationship between betaine and folate metabolism and motivate population-based studies on betaine status in pathologies related to folate deficiency and homocysteine status. Furthermore, because PML tHcy confers increased risk for occlusive vascular disease independent of fasting tHcy, betaine and related metabolites should be included in future studies of homocysteine and cardiovascular risk. Because basal betaine showed a significant relation to ∆PML tHcy as well, the role of betaine could be investigated in epidemiological studies based on biobanks, where PML samples may not be available.

Acknowledgments

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TABLE I. PML Betaine as Predictor of the Increase in tHcy After Methionine Loading at Baseline After 3 Months of Intervention by Multiple Linear Regression*

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Independent variables</th>
<th>β</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>tHcy increase</td>
<td>Age</td>
<td>0.05</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>0.04</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Creatinine</td>
<td>0.19</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Folate</td>
<td>-0.32</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Cobalamin</td>
<td>0.01</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Vitamin B6</td>
<td>-0.26</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>PML betaine</td>
<td>-0.33</td>
<td>0.007</td>
</tr>
</tbody>
</table>

*All variables are included in the model.

tHcy, total homocysteine; PML, post methionine load.